

***In vitro* effect of Levamisole on Rainbow Trout (*Oncorhynchus mykiss*) peripheral blood mononuclear cells**

Efecto *in vitro* del levamisol sobre las células mononucleares de la sangre periférica de trucha arcoíris (*Oncorhynchus mykiss*)

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ABSTRACT

The *in vitro* effect of levamisole (1, 10 and 100 µg/ml) on cells of the innate immune response of rainbow trout (*Oncorhynchus mykiss*) was studied, particularly the activity of natural cytotoxic cells and macrophages present in peripheral blood. Levamisole effect on natural cytotoxic cells was evaluated by their capacity to lyse HL-60 tumor cells while activity of macrophages was evaluated measuring production of reactive oxygen species and nitric oxide. Results indicate that natural cytotoxic cells activity was significantly increased by 1 µg/ml levamisole and that higher doses had no effect. On the other hand, reactive oxygen species and nitric oxide production by macrophages was not increased by any of the levamisole doses used and that the higher dose (100 µg/ml) induced a significant decrease of nitric oxide levels, which might be deleterious for the defensive response of these fish.

KEYWORDS: Innate immunity; reactive oxygen species; ROS; nitric oxide.

RESUMEN

Se estudió el efecto *in vitro* del levamisol (1, 10 y 100 µg/ml) sobre células de la respuesta inmune innata de trucha arcoíris (*Oncorhynchus mykiss*), particularmente la actividad de las células citotóxicas naturales y de los macrófagos presentes en sangre periférica. El efecto del levamisol sobre las células citotóxicas naturales fue evaluado mediante su capacidad para lisar células tumorales HL-60, mientras que la actividad de los macrófagos fue evaluado mediante la producción de especies reactivas del oxígeno y de óxido nítrico. Los resultados indican que la actividad de las células citotóxicas naturales fue incrementada significativamente por 1 µg/ml levamisol mientras que dosis mayores no produjeron este efecto. Por otro lado, la producción de especies reactivas del oxígeno y de óxido nítrico por los macrófagos no fue incrementada por ninguna de las dosis usadas, sin embargo dosis de 100 µg/ml indujeron una disminución significativa en los niveles de óxido nítrico, lo cual podría ser deletéreo para la respuesta defensiva de estos peces.

PALABRAS CLAVES: Inmunidad innata; especies reactivas del oxígeno; ROS; óxido nítrico.

INTRODUCTION

Immunity against “foreignness” appeared early in evolution. Innate immunity, characterized as non-specific, is the first defense strategy and the most widely distributed and present in plants, invertebrates and vertebrates (Heine 2008). With the appearance of the first vertebrates, particularly gnathostomes, a new defensive strategy, the acquired or adaptive immune response (specific and with memory), acting jointly and coordinately with the innate response, appeared (Flajnik & Du Pasquier 2004; Cooper & Alder

2006). Thus, the adaptive immune response is present in most fish and all the rest of vertebrates and, therefore, fish are a key group for comparative studies to unravel the evolution of adaptive immunity (Plouffe *et al.* 2005).

Fish immunity is dependent on innate and adaptive (or acquired) responses (Secombes 1996). Innate immunity, being the first defensive line against microorganisms, is fundamental to avoid infectious diseases (Plouffe *et al.* 2005). Among the cellular components of innate immunity we can include natural cytotoxic cells (NCCs), functionally

homologous to natural killer cells (NK) of mammals (Evans & Jaso-Friedmann 1992; Praveen *et al.* 2006). One of the main functions of NCCs is to eliminate, without the requirement of a previous contact or recognition, intracellularly infected or transformed cells by lysing them or inducing their apoptosis (Praveen *et al.* 2006). Other important effectors of the innate response in fish are macrophages (Secombes 1996). Macrophages destroy pathogenic microorganisms by several mechanisms, including phagocytosis and production of reactive oxygen species (ROS) and nitric oxide (NO) (Secombes 1996; Lundén *et al.* 2002).

The activity of both cell populations (NCCs and macrophages) can be enhanced by a series of compounds with immunostimulatory properties. These compounds, being efficient to increase immunocompetency and resistance to infections, have been used in aquaculture, veterinary and human medicine (Sakai 1999). One of these immunostimulating compounds is levamisole (Kajita *et al.* 1990; Findlay & Munday 2000; Ispir & Yonar 2007). It is a synthetic compound originally used as an anti-helminthic livestock and later on its immunostimulating property was demonstrated, leading to its use with this second purpose in veterinary and human medicine (Sakai 1999). In the aquaculture industry, it is used to increase defensive responses in fish (Findlay & Munday 2000; Ispir & Yonar 2007). *In vivo* results have shown its positive effect on the innate and adaptive responses (Cuesta *et al.* 2002; Kumari & Sahoo 2006; Ispir & Yonar 2007; Maqsood *et al.* 2009).

Hence, the *in vivo* immunostimulating effect of levamisole has been widely studied. Nevertheless, *in vitro* studies in fish and other animals have shown that this compound is immunosuppressive at high doses, negatively affecting the activity of some immunocompetent cells (Siwicki *et al.* 1990; Mulero *et al.* 1998; Li *et al.* 2004). For this reason, the purpose of this study was to evaluate the *in vitro* effect of different doses of levamisole on the activity of natural cytotoxic cells (NCCs) and peripheral blood macrophages, important components of the innate immune response in rainbow trout.

MATERIALS AND METHODS

FISH AND BLOOD SAMPLES

Apparently healthy 10 rainbow trouts (*Oncorhynchus mykiss* Walbaum 1792) of both sexes per group, ranging from 180 to 210 g, were kept at the "Salmones Pangué" pisciculture (Bio Bío Region, Chile) during the summer, under normal conditions of culture. Blood samples were obtained by caudal vein puncture using heparinized syringes after animals were anesthetized with 15 ml of 20% benzocaine (Veterquímica,

Santiago, Chile) in 100 l of water. Blood was transported, at 4° C, to the Laboratory of Immunology, Department of Microbiology, Faculty of Biological Sciences, University of Concepción (Concepción, Chile) arriving in not more than 1 h after been drawn.

ISOLATION AND CULTURE OF MONONUCLEAR CELLS

Since a good source of NCCs and macrophages are the peripheral blood mononuclear cells (PBMCs), peripheral blood was centrifuged at 500 x g during 30 min in ficoll-hypaque (density 1.077 g/ml) (Sigma Diagnostics, St. Louis., MO., USA) to separate the PBMCs (Greenlee & Ristow 1992). After washing three times in sterile saline solution, the viability of PBMCs was assessed using 0.4% trypan blue (Sigma Chemical Co, St Louis MO, USA). Cellular viability exceeded 95% in all samples. PBMCs were finally resuspended at a concentration of 1×10^6 viable cells/ml in RPMI 1640 medium (Gibco BRL, Grand Island NY, USA) supplemented with 10% fetal calf serum (FCS), 100 UI/ml penicillin and 120 µg/ml streptomycin (Sigma Aldrich Inc, St. Louis, MO, USA). Experimental groups received levamisole {(L-)-2,3,5,6-Tetrahydro-6-phenyllimidazo [2,1-b]thiazole} Hydrochloride} (Sigma Chemical Co., St. Louis, MO. USA) at final doses of 1, 10 or 100 µg/ml and were cultured for 24 h in 96 wells microtiter plates at 18 °C. Controls lacked levamisole.

ACTIVITY OF NONSPECIFIC CYTOTOXIC CELLS (NCCs)

After 24 h of culture in the absence or in the presence of 1, 10 or 100 µg/ml levamisole, natural cytotoxic activity was assessed co-culturing PBMCs, which includes NCCs (Greenlee & Ristow 1992), with HL-60 tumor line cells (ATCC, Rockville, MD. USA). Characteristics of HL-60 cells, a myeloid cell line from a patient with promyelocytic leukemia, including morphology as observed by light and transmission electron microscopy, growth kinetics, karyotype, cell surface markers, tumor formation and others were described by Gallagher *et al.* (1979). To measure the lysis of the tumor cells by NCCs, HL-60 cells (kept in RPMI 1640 medium supplemented as above) were previously labeled with 0.036% w/v neutral red (Merck, Darmstadt, Germany) in PBS for 30 min at 37° C, washed three times and adjusted to a concentration of 1×10^6 viable cells/ml in RPMI 1640 supplemented as above. Effector PBMCs were mixed with HL-60 target cells at a 1:10 ratio and co-incubated during 180 min at 15° C. Cytotoxic activity was measured by neutral red release from HL-60 cells lysed by NCCs using a spectrophotometer at 540 nm (Parish & Mullbacher 1983). Assays were done in triplicate.

PRODUCTION OF ROS AND NO BY MACROPHAGES

Reactive oxygen species (ROS) and nitric oxide (NO) produced by macrophages, cellular population included in the PBMCs, was measured. ROS produced after 24 h

stimulation with 1, 10 or 100 µg/ml levamisole and controls were measured as described by Anderson & Siwicki (1993) with modifications. This method is based on the reduction of nitroblue tetrazolium (NBT) by the superoxide anions to the colored compound formazan, which can be spectrophotometrically measured. Briefly, 100 µl of the experimental and control cultures containing 1×10^6 PBMCs/ml were incubated, in Eppendorf tubes, with 100 µl 0.1% NBT (Sigma Chemical Co., St. Louis., MO., USA) for 60 min. Later on, 1 ml N,N-dimethylformamide (Merck, Darmstadt, Germany) was added and the Eppendorf tubes centrifuged for 10 min at 100 x g. Absorbance of the supernatant was measured at 620 nm. Assays were done in triplicate.

Nitric oxide (NO) production after 24 h of culture in the presence of 1, 10 or 100 µg/ml levamisole and controls was determined using a procedure modified from Walsh *et al.* (2006), based on detection of nitrite (NO_2^-) derived from the nitric oxide (NO) by means of the Griess reagent (sulphanilamide, naphthylethylenediamine and phosphoric acid) (Sigma-Aldrich Co., St. Louis., MO., USA). Briefly, from the cell cultures (1×10^6 PBMC/ml) treated with the different concentrations of levamisole, 200 µl were obtained and centrifuged for 10 min at 100 x g and 100 µl of supernatant was collected. The supernatant was incubated for 10 min with 100 µl de 1% sulphanilamide in 5% phosphoric acid, and 100 µl of 0.1% N-naphthylethylenediamine were then added and incubated for 30 additional min. Nitrite was assessed spectrophotometrically at 540 nm in the supernatant. Assays were done in triplicate.

STATISTICAL ANALYSIS

The effect of the different doses of levamisole on the natural cytotoxic activity and production of ROS and NO

was determined using a one-way ANOVA and Tukey's test. Significant differences were considered those with a value of $p < 0.05$.

RESULTS

EFFECT OF LEVAMISOLE ON NCCs ACTIVITY

Natural cytotoxic activity by NCCs was determined by lysis of HL-60 tumor cells. Results indicate that a dose of 1 µg/ml significantly increases cytotoxic activity ($p = 0.039$), but higher doses, 10 or 100 µg/ml, show results not significantly different from controls (Fig. 1).

EFFECT OF LEVAMISOLE ON MACROPHAGES ROS AND NO ACTIVITY

Activity of macrophages was assessed by the production of ROS and NO after 24 h of incubation with levamisole. In this work performed *in vitro*, ROS production was not affected by any of the levamisole doses used (Fig. 2). On the other hand, *in vitro* levamisole effect on NO production shows that 1 or 10 µg/ml had no effect on NO production but 100 µg/ml induced a significant reduction in the production of this molecule ($p=0.035$) (Fig. 3).

DISCUSSION

Levamisole is a synthetic compound with immunostimulatory properties (Sakai 1999). It has been reported that in salmonids, such as *Salmo salar* and *Oncorhynchus mykiss*, *in vivo*, it increases immunity (Kajita *et al.* 1990; Findlay & Munday 2000; Ispir & Yonar 2007). Nevertheless, some reports indicate that, *in vitro*, high doses of levamisole induce immunosuppression, especially on the antibody forming cells (AFCs), macrophages and ROS production

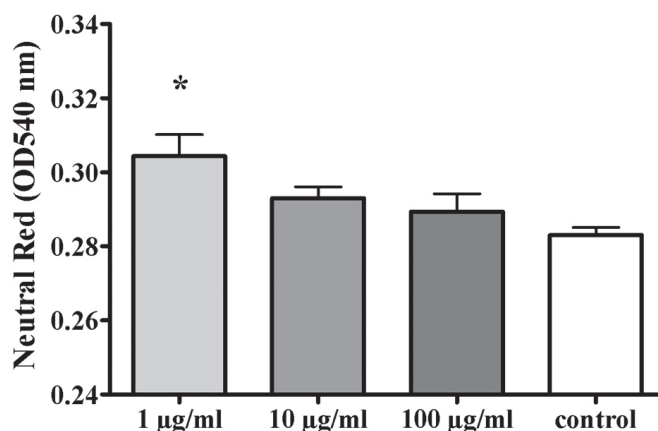


FIGURE 1. Effect of different doses of levamisole (24 h treatment) on the natural cytotoxic activity by trout peripheral blood mononuclear cells. Bars indicate mean \pm standard deviation. * Statistically significant difference ($p < 0.05$).

FIGURA 1. Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la actividad citotóxica natural de las células mononucleares de sangre periférica de trucha. Las barras indican el promedio \pm desviación estándar. * Diferencias estadísticamente significativas ($p < 0.05$).

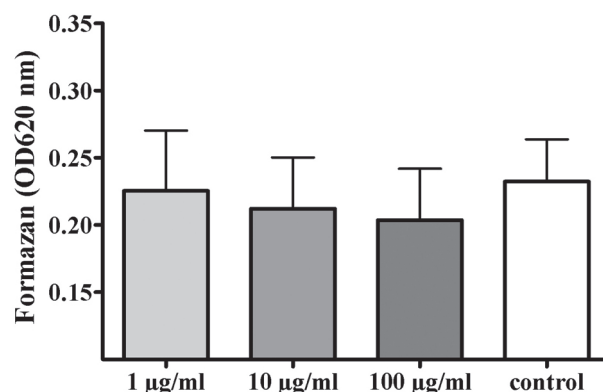


FIGURE 2. Effect of the different doses of levamisole (24 h treatment) on the production of reactive oxygen species (ROS) by trout peripheral blood mononuclear cells. Bars indicate mean \pm standard deviation.

FIGURA 2. Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la producción de especies reactivas del oxígeno (ROS) por las células mononucleares de sangre periférica de trucha. Las barras indican el promedio \pm desviación estándar.

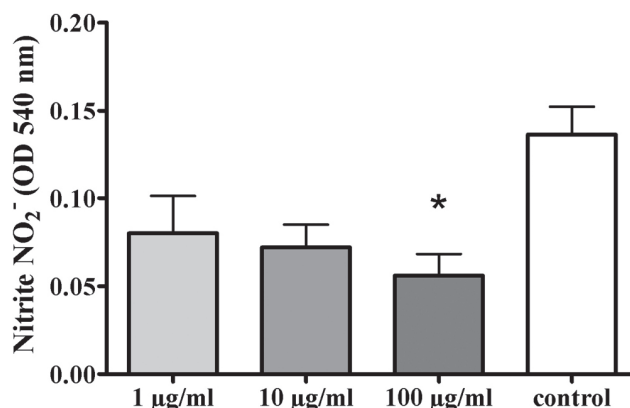


FIGURE 3. Effect of the different doses of levamisole (24 h treatment) on the production of nitric oxide (NO) measured as Nitrite (NO₂⁻) by trout peripheral blood mononuclear cells. Bars indicate mean \pm standard deviation. * Statistically significant difference ($p < 0.05$).

FIGURA 3. Efecto de diferentes dosis de levamisol (24 h de tratamiento) sobre la producción de óxido nítrico (NO) medido como Nitrito (NO₂⁻) por las células mononucleares de sangre periférica de trucha. Las barras indican el promedio \pm desviación estándar. * Diferencias estadísticamente significativas ($p < 0,05$).

(Siwicki *et al.* 1990; Mulero *et al.* 1998; Li *et al.* 2004). For this reason, this work studies the *in vitro* effect of different levamisole doses on the activity of natural cytotoxic cells (NCCs) and macrophages, important components of the innate immune response, in trout.

Activity of NCCs was evaluated by their cytotoxic activity against HL-60 tumor cells while activity of macrophages was evaluated by the production of ROS and NO. NCCs and macrophages were obtained by isolation of PBMCs. PBMCs include lymphocytes, NCCs and macrophages (Yang *et al.* 1994). Nevertheless, given the conditions of the assays performed, only NCCs, and not other cellular populations, could exert cytotoxic activity and only macrophages present in the culture were able to produce ROS and NO for defensive purposes (Secombes 1996; Lundén *et al.* 2002).

The effect of levamisole on NCCs has been reported in various fish species (Findlay & Munday 2000; Cuesta *et al.* 2002; Choi 2004), including rainbow trout (Kajita *et al.* 1990; Ispir & Yonar 2007). Our results show a significant increase of trout NCCs activity stimulated *in vitro* for 24 h with 1 µg/ml levamisole (Fig. 1). These results are in agreement with those reported by Cuesta *et al.* (2002) for the gilthead seabream (*Sparus aurata*) and by Choi (2004) for the Japanese flounder (*Paralichthys olivaceus*) showing that *in vitro* levamisole doses ranging from 0.001 to 0.1 µg/ml (doses under the lowest used in this work) also significantly increased the activity of NCCs. Hence, the positive effect of low doses of levamisole on NCCs activity is confirmed in different species of teleosts. Furthermore, we can report that high doses of levamisole (up to 100 µg/ml), which might be deleterious for other defensive functions, such as

the antibody forming cells (AFCs), macrophages and ROS production (Siwicki *et al.* 1990; Mulero *et al.* 1998; Li *et al.* 2004), do not inhibit natural cytotoxic activity (Figure 1). In brief, considering the role played by NCCs in the defense of an organism, we can conclude that low doses of levamisole are favorable in the immune response against tumor cells and intracellular pathogens and that high doses would not negatively affect NCCs.

Besides evaluating the effect of levamisole on trout NCCs, we also studied its effect on macrophages, particularly on ROS and NO production by these cells. When evaluating the *in vitro* levamisole effect on ROS production by macrophages, contrary to what happened with NCCs activity (Figure 1), there was no ROS increase with any of the three doses used (Figure 2). Our results are similar to those reported by other authors, such as Mulero *et al.* (1998), reporting that *in vitro* doses below 1 µg/ml do not increase ROS production in the gilthead seabream (*S. aurata*). But, they showed a significant decrease in ROS production after 48 h of stimulation with 1 µg/ml levamisole, a time span not evaluated in this work. Also, Li *et al.* (2004) reported an *in vitro* decrease in ROS production in the hybrid striped bass (*Morone chrysops* and *Morone saxatilis*) after treatment with 1000 µg/ml levamisole for 24 h, a tenfold increase if compared with the highest dose studied in this work. These results suggest that *in vitro* treatment with levamisole does not produce the same increase in ROS production by macrophages observed *in vivo* (Kumari & Sahoo 2006; Ispir & Yonar 2007).

In vertebrates in general, activated T lymphocytes produce a series of cytokines able to regulate the activity of NCCs and macrophages (Bird *et al.* 2006). Thus, we cannot discard that levamisole *in vivo* activates T lymphocytes which, in turn, exert their regulatory effect on NCCs and macrophages. *In vitro* work with PBMCs might not provide the same cellular interactions present *in vivo*, thus explaining the different results obtained when working *in vivo* or *in vitro*, that is to say the presence or absence, respectively, of ROS increase after levamisole stimulation.

We have been unable to find other reports evaluating nitric oxide (NO) production by teleost macrophages after levamisole stimulation. Our results indicate that levamisole doses of 1 and 10 µg/ml do not affect nitric oxide production, while 100 µg/ml significantly reduced production of this compound (Fig. 3). This suppression might be related to the already described negative effect of high *in vitro* levamisole doses on some parameters of the immune response (Siwicki *et al.* 1990; Mulero *et al.* 1998; Li *et al.* 2004).

Since *in vivo* low doses of levamisole exert a positive effect absent in *in vitro* experiments, we can support the idea that

macrophages may not be the direct target for levamisole effect, but other immunocompetent cells might respond *in vivo* to levamisole and secrete cytokines which, in turn, will signal macrophages. Some reports suggest that T lymphocytes might be targets for levamisole action (Tizard 2009). For this reason, it becomes necessary to determine if trout T lymphocytes in fact regulate the activity of NCCs and macrophages. If so, studies involving levamisole might help to comprehend the regulatory role of T lymphocytes on the activity of NCCs and macrophages, the interaction of immunocompetent cells in teleost fish and, therefore, to search ways to better stimulate the immune response of these animals against infectious diseases.

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